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Application News

A Biological Model of the Ageing Metabolome Reveals Potential Clinically Relevant Biomarkers

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User Benefits

- Applying an LC/QTOF method with HILIC separation to an untargeted metabolomics workflow to identify metabolic profiles in biological cell extracts and cell culture media samples.
- A flexible workflow for untargeted metabolomics analysis generates data compatible for analysis using LabSolutions Insight Explore™ as well as highly cited third-party software solutions for metabolomics such as MS-DIAL and MetaboAnalyst.
- DIA-MS/MS analysis enables library searching of all detected metabolites in all samples in an untargeted approach.

■ Introduction

Ageing can have a profound effect on the metabolome through mechanisms including mitochondrial dysfunction, genomic instability, altered intercellular communication and cellular senescence. In this research, serially passaged human foreskin fibroblasts (HFF-1 cells) and their media have been considered as a biological model to increase our understanding of the ageing metabolome. Untargeted metabolomic analysis of cell and media extracts has revealed potential clinically relevant biomarkers of ageing.

An end-to-end workflow for untargeted metabolomics analysis is presented based on an untargeted HILIC-DIA-MS/MS method for data acquisition, MS-DIAL and MetaboAnalyst for data analysis and LabSolutions Insight Explore for metabolite identification. Fig. 1 highlights extracted ion chromatograms for polar metabolites in a cell extract analysed using this analytical method.

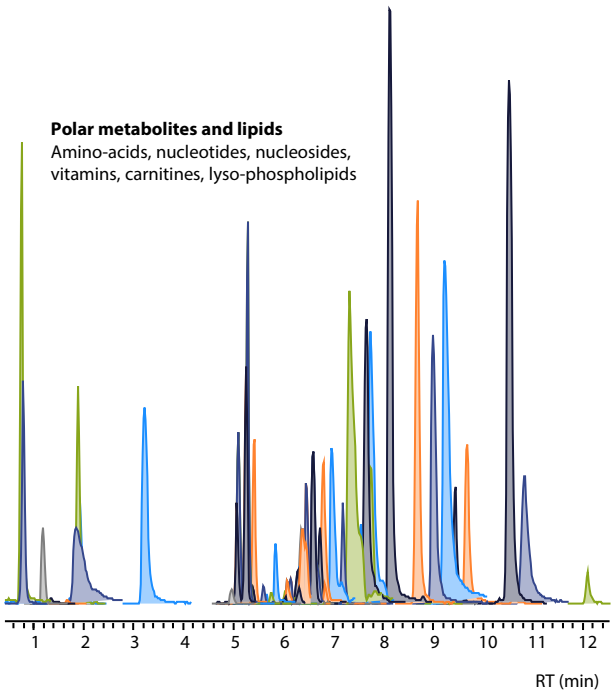

Fig. 1 Mass chromatograms for a panel of polar metabolites in a cell extract separated using a HILIC phase (precursor ion ±5 ppm).

■ Sample analysis

A biological model of cell senescence considered the comparison of HFF-1 cells and media from an early passage versus a later passage representative of a senescent state. Extracts from HFF-1 cells and culture media at passage 3 and passage 20 were analysed with a high-resolution untargeted metabolomics LC/QTOF method (LCMS-9030 Q-TOF). The analysis was performed using the analytical conditions described in Table 1. Samples were analysed separately for cells and media. Each batch was analysed in a randomised order.

Table 1 Analytical conditions

UHPLC:	Nexera X2
Column	Shim-pack Velox HILIC (2.1x100 mm, 2.7 µm, PN: 227-32025-03); column maintained at 40 ˚C
Flow Rate	0.3 mL/min (0-13 min); 0.4 mL/min (13-17 min); 0.3 mL/min (17-18 min)
Mobile Phase	A: Water + 10 mM ammonium formate + 0.1% formic acid B: Acetonitrile:water [92:8] + 10 mM ammonium formate + 0.1% formic acid
Rinse (R0/R1/R2)	IPA/MeOH/MeCN/H_2O (1:1:1:1) + 1% formic acid
Gradient	98% B (0-1.5 min); 65% B (11 min); 10% B (12.5-14 min); 98% B (14.25-18 min)
Injection	0.5 µL; autosampler temperature 4 ˚C
Mass Spectrometer:	**LCMS-9030**
Interface	300 ˚C, 1.5 kV (ESI+) / -3.0 kV (ESI-)
Heat block, DL	400 ˚C, 250 ˚C
Gas flow rates	Nebulizing gas 3 L/min; Heating gas 10 L/min; Drying gas 15 L/min
CID gas pressure	230 kPa
MS	TOF-MS (*m/z* 60-1000)
MS/MS	27 DIA-MS/MS (*m/z* 40-1000; precursor isolation 35 Da; collision energy spread 5-55 V
Total cycle time (MS and MS/MS)	0.991 seconds

■ Data processing; feature detection and alignment

MS-DIAL, a universal program for untargeted metabolomics, was used to find and align features across all samples. Default processing parameters were applied to data files without any data conversion (MS-DIAL reads LabSolutions™ raw data files directly). MS-DIAL can be applied to process data from both data-dependent or data-independent acquisition data types automatically without supplying additional information on data structure.

As shown in Fig. 2, MS-DIAL allows the easy navigation of samples and features. Selecting any peak in the 'peak spot viewer' shows the extracted ion chromatogram for that feature in the sample highlighted in the 'file navigator'. The MS spectrum for the feature in the highlighted sample is displayed in the 'survey scan (MS1) spectrum'. In this case, data were acquired using DIA-MS/MS so MS-DIAL performs deconvolution; the deconvoluted MS/MS chromatogram can be visualised for each feature as shown.

Fig. 2 MS-DIAL was used to process LabSolutions raw data files in positive and negative ion mode separately, to find and align features that behaved as chromatographic components. In this example, cell and media extracts were aligned together for each ion mode.
(In this application, MS-DIAL was used to find and align features only, and not for metabolite annotation).

■ Data analysis; feature filtering and statistical analysis

In this ageing metabolomics study, data were exported from MS-DIAL and filtered to retain features found in at least 80% of samples in the passage 3 or the passage 20 group. Features were filtered for cells or media separately. Feature arrays from positive and negative ion mode were combined for each sample type (cells or media) for statistical analysis.

MetaboAnalyst was used for statistical analysis of the combined feature arrays from positive and negative ion mode. Missing values were automatically detected, and the default of replacing missing values by 1/5 of min positive values of their corresponding variable was applied. Interquartile range filtering was applied within the software to remove near constant features (default of 40% applied).

To find statistically significant differences between the early and later passage groups of cells or media (six samples of each), volcano plot analysis was performed in MetaboAnalyst considering a fold change >2 and p-value <0.05 in passage 20 vs passage 3 samples. The volcano plot from the analysis of cell extracts is shown in Fig. 3.

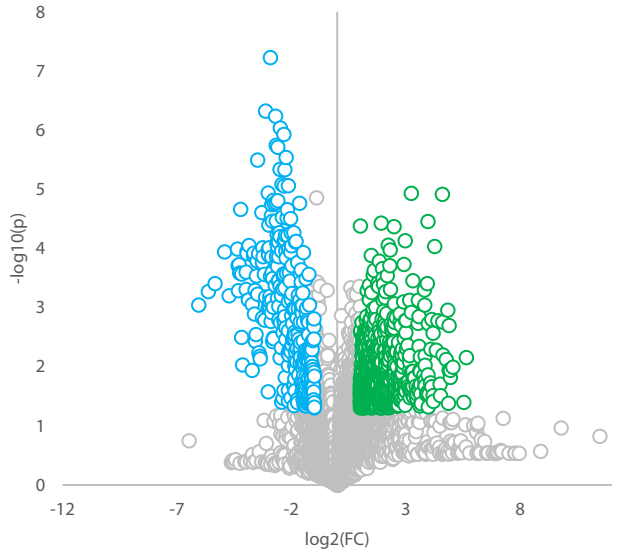

Fig. 3 Volcano plot of features detected using HILIC-DIA-MS/MS (positive and negative ESI combined), that were significantly increased (green) or decreased (blue) in cell extracts from later passage (20) compared to earlier passage (3) representative of a cell senescence model (fold change >2, p<0.05).

■ Metabolite identification

Commonly used open-source software applications for data processing cited in literature such as MS-DIAL, MZmine and XCMS offer some internal tools for annotation. However, this application used LabSolutions Insight Explore software, due to support for the conversion of external libraries such as MassBank, HMDB and NIST, as well as including a flexible editor to create user specific libraries from authentic standards.

All significant features from volcano plot analysis were considered for identification. To identify or annotate metabolic features of significance, a processing method was set up in LabSolutions Insight Explore software, considering the detected *m/z* and retention time for each detected and aligned peak from MS-DIAL. Library searching was performed to identify metabolites at Metabolomics Standards initiative (MSI)[1] level 1-comparison to authentic standards or level 2-comparison to external resources such as MassBank or HMDB. Within LabSolutions Insight Explore, up to 25 hits can be returned for each feature in each sample by searching up to 5 different libraries simultaneously.

Fig. 4 highlights the identification of taurine from the negative ion mode analysis of cell extracts, which was matched to an authentic standard as well as the HMDB with high reporting confidence. The 'compound list' details all negative ion mode features from MS-DIAL that were found to be significant in volcano analysis along with the measured *m/z* and RT as exported from MS-DIAL. For the highlighted compound (in this case #205 with measured *m/z* 124.0071 and Rt 4.922), the 'sample results' are displayed for each sample. In the example below, the sample name, compound name from the top library hit (Lib. Compound Name) and associated similarity index (Lib. SI) score from a dot-product based reverse search are displayed. The integrated peak area derived for this feature in each sample is also shown.

Within the 'compound details' pane, the extracted ion chromatogram for the selected compound in the selected sample is displayed along with the associated MS1 spectrum and DIA-MS/MS spectrum, mirror reflected against the top library hit. The 'library hits' pane shows all hits derived for this search; the analyst may select a different hit from this list to report if desired.

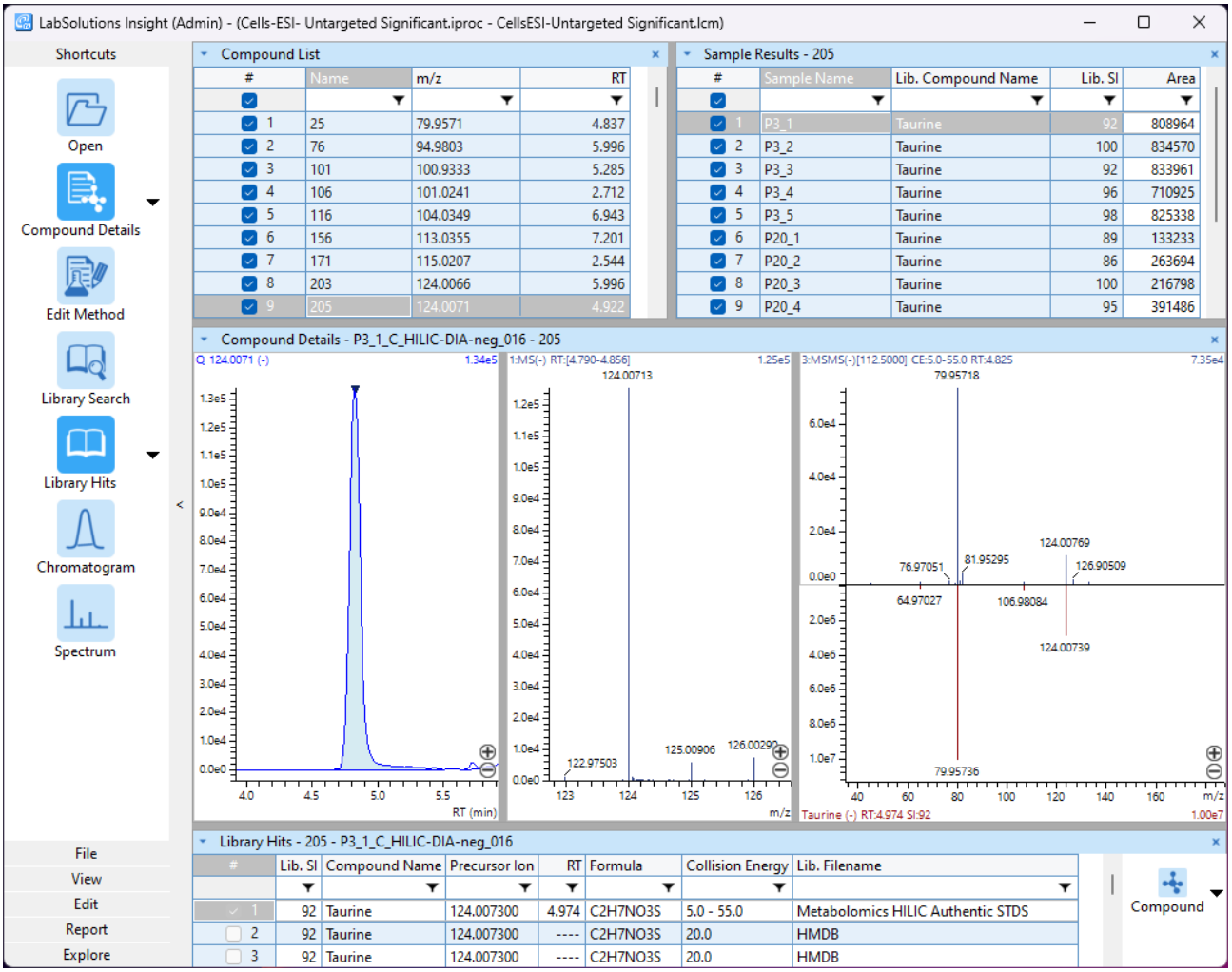

Fig. 4 Features found to be significant by volcano plot analysis were considered for identification / annotation. LabSolutions Insight Explore software was used to search significant features against up to 5 libraries simultaneously. In this example, the MSI level 1 identification of taurine by comparison to MS/MS from authentic standard in negative ion mode is presented. Taurine was found to be significantly reduced in passage 20 cell extracts compared to passage 3 cell extracts.

■ Results

Following automated data processing in MS-DIAL, statistical analysis in MetaboAnalyst and metabolite identification in LabSolutions Insight Explore, several metabolites were identified as being statistically significantly different between passage 3 and passage 20 cells (Fig. 5) and media (Fig. 6). The number in brackets next to each identification denotes the MSI level of identification.

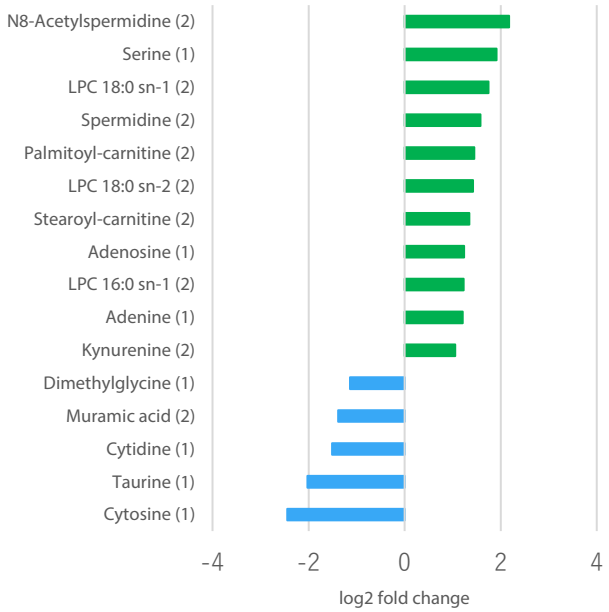

Fig. 5 Bar-chart highlighting log2 fold changes for significant metabolite features in cell extracts identified by MS/MS spectral library searching to MSI level 1 or 2 (denoted after each metabolite name in brackets). Metabolites elevated with senescence correspond to an increase in passage 20 cells compared to passage 3 cells.

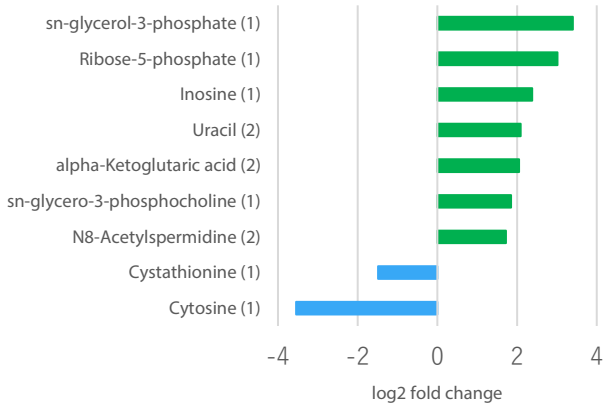

Fig. 6 Bar-chart highlighting log2 fold changes for significant metabolite features in media extracts identified by MS/MS spectral library searching to MSI level 1 or 2 (denoted after each metabolite name in brackets). Metabolites elevated with senescence correspond to an increase in passage 20 media compared to passage 3 media.

■ Conclusion

An end-to-end workflow has been applied to the untargeted metabolomics analysis of cell and media extracts using a high-resolution HILIC-DIA-MS/MS method for data acquisition, MS-DIAL and MetaboAnalyst for data analysis and LabSolutions Insight Explore for metabolite identification. The workflow has been applied to explore the ageing metabolome by comparing HFF-1 cells and their media at passage 3 and passage 20.

Samples were analysed in both positive and negative ion mode and data were processed to find and align chromatographic features in MS-DIAL. MetaboAnalyst was applied to analyse statistically significant differences in the early and later passage cells or media extracts using volcano plot analysis. LabSolutions Insight Explore was used to identify metabolites.

Untargeted metabolomic analysis of cell and media extracts has revealed potential biomarkers of ageing.

■ References

1. Spicer, R., Salek, R. & Steinbeck, C. (2017) A decade after the metabolomics standards initiative it's time for a revision. Sci Data 4, 170138.

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